SOME EFFECTS OF NUMBERS PRESENT ON THE RATE OF CLEAVAGE AND EARLY DEVELOPMENT IN ARBACIA

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Frank and Kurepina (1930) reported accelerated development for sea urchin eggs, species not given, when 10 to 20 eggs were present per drop as compared with the rate when only one or two eggs were present. They used hanging-drop cultures and determined the relative degree of development at varying times after fertilization. Apparently they did not examine early cleavages. They interpreted their findings as being the result of the action of mitogenetic rays. Allee and Dr. J. R. Fowler began the exploration of this problem at Woods Hole during the summer of 1931 and obtained preliminary results for *Arbacia punctulata* which indicated that fertilized eggs left standing in drops of varying size for 16 hours or longer showed a decided tendency toward faster development in the more crowded as compared with the less crowded populations. At times, however, isolated eggs would develop as rapidly and as perfectly as did those in the somewhat crowded cultures. In these preliminary and hurried experiments there was no steady difference in the time to 50 per cent cleavage for the first two cleavages which were the only ones studied.

The present authors took up this problem in the summer of 1934. The methods of handling the sea urchins, of procuring eggs and sperm and of fertilization were based closely on the directions given by Just (1928). Usually freshly collected animals were used one to two days after being delivered in our laboratory. Later in the season eggs and sperm were obtained from sea urchins which had been collected for some time and, by being kept in the laboratory, still retained their sexual products when those collected daily were mostly spent.

Specifically we dried the animals on paper towels, meantime pressing the spines out of the way and breaking several loose from the body. The urchins were then opened by cutting with scissors around the peristome; Aristotle's lantern was removed with tweezers and the edge of the peristome shell was broken somewhat. The body fluid was shaken out and the animals thus prepared were placed aboral side down in a

clean, dry Syracuse watch-glass. Normally eggs were soon shed by one or more of the females. These were removed after extrusion by pipetting through washed new cheese cloth into finger-bowls containing filtered sea water and were decanted through several changes of similar sea water. Shed sperm were selected according to Just's criteria, covered and left until needed. One drop of the "dry" sperm was diluted in 10 cc. of filtered sea water in a Syracuse watch-glass. At first this was used almost immediately but beginning with the hanging-drop experiments diluted sperm was allowed to stand for 2 to 3 minutes before being used to inseminate eggs. Fertilization was accomplished by adding two (or sometimes one, or three) drops of this suspension to the eggs in 250 cc. of sea water in a finger-bowl.

Early and late in the season when the *Arbacia* were not shedding readily, the ovaries were shaken over cheesecloth to obtain eggs. Late in the season, on a few occasions sperm were pipetted from ripe appearing testes which, however, were not shedding normally. Trial fertilizations came to be the rule; usually evidence from the fertilization membrane stage proved trustworthy. Relatively few experiments had to be discarded because of low percentage of cleavage. Eggs from one female only were used in any given comparison; usually eggs from one female sufficed for all experiments made in any given half-day. Late in the season eggs from a "good" female which had been opened about 9:30 A.M. were used for inseminations up to about 3:00 P.M. The fertilized eggs were allowed to stand 5 minutes and were then transferred to experimental conditions. All experiments were eliminated from consideration which did not show at least 95 per cent cleavage in the sparse populations and 90 per cent cleavage in the more densely populated drops. Usually the cleavage was well above 95 per cent in both.

When 90 or 110 per cent sea water was used in the experiment, the eggs were usually fertilized in this medium. At times, with these solutions, the eggs were fertilized in normal filtered sea water and centrifuged at the lowest speed shown by a power centrifuge for 15 seconds; this sufficed to throw them to the bottom. The supernatant sea water was poured off and the eggs were gently washed into the appropriate experimental medium.

After the first few days, initial handling of eggs and sperm was all done by Evans; two stop-watches were started by Allee at the moment of dropping the second drop of sperm suspension into the water containing eggs. All isolations and group transfers were made by Allee under a low power dissecting binocular. Typically, after standing five or six minutes, a part of the fertilized eggs were pipetted to a clean

Syracuse watch-glass recently rinsed with sea water. Using a hæmocytometer tube in which each of the ten units on the tube proper equaled 2 cu. mm., the approximate number of eggs needed were transferred to the experimental slides in drops of the selected size. Early in the work these transfers were made in the open air, sometimes, unfortunately, before open windows. Later all transfers were made to experimental slides temporarily housed in a moist chamber lined with wet filter paper. The separation was usually completed by 15 minutes after fertilization.

Cleaning

After the first few days, cleaning of glassware was done by Evans. The Syracuse watch-glasses into which the eggs and sperm were shed and the finger-bowls in which the eggs were washed and fertilized and all pipettes were washed in running cold tap water and boiled for at least 10 to 15 minutes. They were usually drained dry on paper towels. Occasionally when needed immediately they were dried with clean cotton towels. The hæmocytometer pipette used in isolations was similarly treated except that at times between isolations of different lots of eggs on the same half-day, it might be rinsed in fresh water and allowed to stand full of fresh water for as long as possible before the next transfer; frequently even under these conditions, this pipette was boiled. The eggs used in successive experiments were fertilized at least 15 minutes apart, hence any contamination could be easily recognized and discounted; very few occurred.

The glass slides were washed in warm soapy water to remove the vaseline and vacuum grease used in making seals. They were thoroughly rinsed in running tap water and were rinsed again in hot water and were usually boiled. They were polished with clean cotton towels.

Temperature

No attempt was made to control the temperature of the water in which the fertilizations took place. This water was usually the normal sea water freshly drawn from the laboratory tap, filtered and left standing in a large Florence flask. It was usually somewhat below air temperature at the time of fertilization. All observations were made in a room having north light only, hence there was no direct sunlight to interfere with room temperatures.

The small separate drops must have approximated air temperatures in a short time. These ran no higher than 25.5° at any time and were usually between 23–25°. When the air temperature was 20° or lower, the whole cleavage test was made at that temperature. In the hypertonic sea water experiments (110 per cent), room temperatures were

also used. At other times the experimental slides were placed in cooling chambers for as much time as possible between the setting up of the experiment and the second cleavage. These chilling chambers varied from a rudely insulated makeshift refrigerator to an elaborately regulated chamber. They were usually held at 17–19° and normally were fairly constant for any given experiment. In one experiment the temperature went as low as 16°. An attempt was made to arrange the temperature so that first cleavage would come after some fifty minutes and second cleavage after about eighty-five minutes. Isolations were completed in about fifteen minutes and the slides were placed immediately in the cooling chamber, if that was the indicated procedure. Except in early experiments, dense and sparse populations were on the same slide and were accordingly subjected to the same temperatures throughout an experiment; hence more exact temperature regulation was not needed.

Test for Accuracy of Determination of Fifty Per cent Cleavage

In our first experiments we undertook to determine cleavage on living eggs. Accordingly we determined the relation between the time recorded by one of us for 50 per cent cleavage in living eggs, using our usual technique, as compared with the time similar eggs cleaved as determined by spaced killings of eggs kept under the same conditions. The mean error of determination of 50 per cent cleavage in eight cases was 10 ± 3 seconds.

Observations

The following experimental conditions were used and in this order:

Paraffined Slides.—Microscope slides with a shallow hollow ground cell were coated with paraffin. Two grades were used. The first had a relatively low melting point. The second was the highest grade, highest melting-point paraffin available in Woods Hole. There was no marked difference in the results obtained with the two grades. Melted paraffin was painted on the slides just before they were to be used and was removed, preparatory to washing them, after each experiment. One drop of water containing eggs was placed in the center of each depression and covered by a thin, somewhat curved watch-glass vaselined around the edge to make a small moist chamber. Typically 12 to 14 such slides made an experimental set. One had a drop containing approximately 100 to 200 eggs. Another had approximately half that number. The remainder held about two each. One person followed the cleavage of the two denser populations under a low power of a compound microscope while the other, seated nearby, followed it for the

sparse populations. After the first experiment, the drops used contained 10 cu. mm.

These drops rounded into bead-like objects. Eggs near the periphery were sometimes difficult to see clearly. The slides which held the more densely populated drops were in the fingers of the observer more than was any one of the slides holding the sparser populations. This may have caused temperature differences between the two lots such as would favor the more rapid cleavage of the eggs in the former.

In the six experiments (see Table II) which meet the percentage of cleavage requirements, the time to 50 per cent first cleavage was 0.93 minutes and to second cleavage 2.17 minutes less in the denser populations of eggs than in the accompanying sparser populations. The statistical probabilities are 0.3 and 0.129 respectively. This means that neither of these differences are statistically significant.

Indications appeared that the paraffin retarded development. This was tested in two direct experiments in which approximately 21 hours after fertilization, the mortality was higher in the paraffined slides, particularly those with sparse populations. Accordingly this experimental method was discarded.

Clean Glass Slides.—The technique used resembled that just described except that no paraffin was used. With both of these sets of slides, it took about one minute to examine the lot of sparsely populated slides. Hence, to be significant, differences needed to be more than one minute provided all of the sparsely populated slides were to be inspected each time. Frequently as the critical time approached this was not the case since some of the slides would have all the few eggs present cleaved and could be discarded for the time being. In all, eight paired comparisons were made under these conditions. These showed a mean time to 50 per cent cleavage which averaged 1.53 minutes less time for the more crowded populations for the first cleavage and 2.2 minutes less for the second cleavage than for accompanying sparse populations (see Table II). These have statistical probabilities of 0.068 and 0.036 respectively. The latter is within the range of statistical significance as commonly interpreted.

Hanging Drops.—A central drop with from 11 to 1,000 eggs was surrounded by five similar drops holding in all from 6 to 28 eggs, usually about 10. As in the preceding series, the size of drops was kept uniform at 10 cu. mm. The drops were fenced from each other by vaseline lines to prevent their flowing together. Each slide, so arranged, was inverted over a salt cellar type watch-glass with ground glass upper surface which was vaselined to seal. A small amount of water in the bottom made an efficient moist chamber. This technique put sparse and

dense drops under the eye of the same observer and on the stage of the same microscope. About one minute was consumed in making one complete inspection as cleavage approached 50 per cent. As elsewhere, if the 50 per cent cleavage point was reached in both dense and sparse populations during the same cycle of observations both were recorded as reaching this stage at the same time. The eggs in these hanging drops were located in the surface film exposed to unknown stresses. This constitutes the chief objection to the method.

Fifteen experiments which met standard conditions gave a mean time to 50 per cent first cleavage of 0.57 minutes faster in the more sparsely populated drops and to 50 per cent second cleavage of 0.24 minutes for the same eggs. These differences have a statistical probability of 0.215 and 0.84 respectively and accordingly the results have no statistical significance (see Table II).

Assembled Moist Chambers.—The assembled moist chambers consisted of a glass base 4 cm. sq. etched in the center with lines 1 mm. or less apart to form squares which aided in counting the denser populations of eggs. The vertical wall was formed by a glass ring ground on both surfaces. This ring was about 3 cm. in diameter and 4 mm. high. It was sealed to the base with vacuum grease and a plain glass cover was sealed on with vaseline. The base usually had vaselined lines to keep drops from running together. In much of the work, the vaselined lines were arranged in circles which limited the surface of the drops to about the same area. In one type of experiment, the denser population was placed in the center; the five surrounding drops held normally two eggs each; in three cases there were as many as 15 to 23 eggs in one of these sparsely populated drops. The time consumed in making a complete cycle of observations was about that with the preceding methods. Later only two drops were used, one with a dense population which might run up to an estimated 8,000 in 20 cu. mm. Nearby, perhaps connected with the denser lot of eggs, perhaps isolated, would be a similar drop with a sparse population. The time from the determination of 50 per cent cleavage in the denser population until a count of cleavage could be made in the sparser one was usually 15 seconds or less. All the later experiments were made using this last method.

The seals on such assembled moist chambers had to be carefully made to avoid evaporation. These seals allow for ease of cleaning and the possibility of rapidly changing clouded covers. Evaporation from the experimental drops was frequently lessened by the addition of unoccupied drops of sea water in the free spaces. Even so, with the small drops and the change of covers made necessary by their clouding on

removal from cooling chambers (see below), some evaporation must have taken place. It is difficult to determine whether the evaporation is equally distributed through these small drops. The drop isolated first, other conditions being equal, should show the greatest amount of evaporation. There is evidence that hypertonicity is a potent factor in retarding the time of cleavage of echinoderm eggs and since in many of these experiments the eggs in the more densely populated drop tended

TABLE I

A summary of time to 50 per cent cleavage with two connected or closely associated drops, one containing a very dense and the other a sparse population. All were tested in normal sea water.

Crowded Drops				Sparsely Populated Drops						
No. Eggs	Minutes to Fifty Per Cent Cleavage		Per-centage Cleaved	No. Eggs	No. Fol-lowed	Minutes to Fifty Per Cent Cleavage		Per-centage Cleaved	Cu. Mm. in Drop	Tempera-ture ° C.
	I	II				I	II			
3500	63.25	94.25	98+	17	17	64.83	97.50	100	10	18–25
4000	61.00	94.50	99+	130	28	62.25	96.67	99+	10	16–24
1600	67.83	100.04	99+	250	24	70.67	104.41	99+	10	16–24
2100	68.33	101.25	99+	11	11	70.75	103.41	100	10	16–24
2000	71.83	105.00	99+	41	21–30	74.00	108.08	100	10	16–24
1800	55.41	84.00	100	40	10	56.17	86.58	100	20	21–21.5
4000	52.17	80.50	100	800	10	60.25	90.25	100	20	21–21.5
5000	62.91	95.75	99	600	16	64.25	97.75	100	40	19
2500	58.67	—	99	800	16	61.00	—	100	40	19
6000	62.33	96.50	96	15	12	63.75	99.08	100	20	19–20
8000	61.25	95.00	96	17	12	63.25	97.25	100	20	19–20
6500	61.58	95.25	95	14	12	61.83	96.25	100	20	19–20
2800	61.33	94.58	95	23	12	61.17	94.83	100	20	19–20
3831	62.145	94.72	98	212	16	64.174	97.67	100−	19	

to cleave first, these were isolated first. Here and elsewhere whenever it was impossible to set up conditions exactly equivalent for dense and sparse populations, the sparser population was favored. This necessitated the isolation of the dense group last when hypotonic sea water was used, since under such conditions the longer the drops stood exposed to air, the more nearly they approached normal concentration.

Similar experiments were performed with normal sea water, with 110 per cent and with 90 per cent sea water. With the 110 per cent sea water no cooling was used to slow down development.

The results obtained with the two drop technique are given in some detail in Table I and all the work done with the assembled moist chamber

is summarized in the last four lines of Table II. In all these cases the time to 50 per cent second cleavage was significantly less for the more crowded eggs. The decreased time ranged from 2.17 to 3.16 minutes and the statistical significance from 0.0134 to 0.0002. As usual, there was less difference in the time elapsed before 50 per cent first cleavage. With 90 per cent sea water the results were slightly negative

TABLE II

A summary of differences in time to 50 per cent first (I) and second (II) cleavage with dense and sparse populations of Arbacia eggs.

No. of Experiments	Method	Mean Difference in Minutes		Statistical Probability	
		I	II	I	II
6	Paraffin	0.93	2.17	0.3*	0.129*
8	Glass slides	1.53	2.2	0.068*	0.036
15	Hanging drops	−0.57	−0.24	0.215*	0.84*
21–23	Moist chamber	0.54	2.7	0.194*	0.002
17–18	Do. 110 per cent	1.9	3.16	0.0084	0.0002
10	Do. 90 per cent	−0.03	2.17	0.67*	0.0134
12–13	Do. connected drops	2.03	2.95	0.0036	0.0014
True means and combined significance		0.88	2.23	0.053	0.0016

* Results taken alone are not statistically significant.

(0.03 minutes, probability 0.67). In two other sets of experiments this time difference increased to 1.9 and 2.03 minutes earlier cleavage for the more crowded drops with statistical probabilities of 0.0084 and 0.0036 respectively. The fourth comparison made (line 4, Table II) gave positive results which, however, lack statistical significance for first cleavage although there is good significance for the second cleavage.

The use of two connected drops, one with a dense and the other with a sparse population of eggs, sometimes permitted the observers to determine the time to 50 per cent cleavage in different regions with different densities. Two such determinations (in which Allee was observing and Evans was recording) are summarized in Fig. 1.

Averages of the time to 50 per cent first or second cleavage for the experiments summarized in Table II have lessened value because of the varieties of techniques used. The mean differences are based on individual paired experiments in any one of which the difference in density of the egg population was the only factor known to vary. If the true mean, considering the number of individual tests, is taken, there is a mean difference in time to 50 per cent first cleavage of 0.88 minutes

with the eggs in the denser populations cleaving first. This has a statistical probability of 0.053 which is slightly above the conventional limit of statistical significance. By second cleavage, this difference has increased to a mean of 2.23 minutes and the statistical probability has increased to 0.0016.

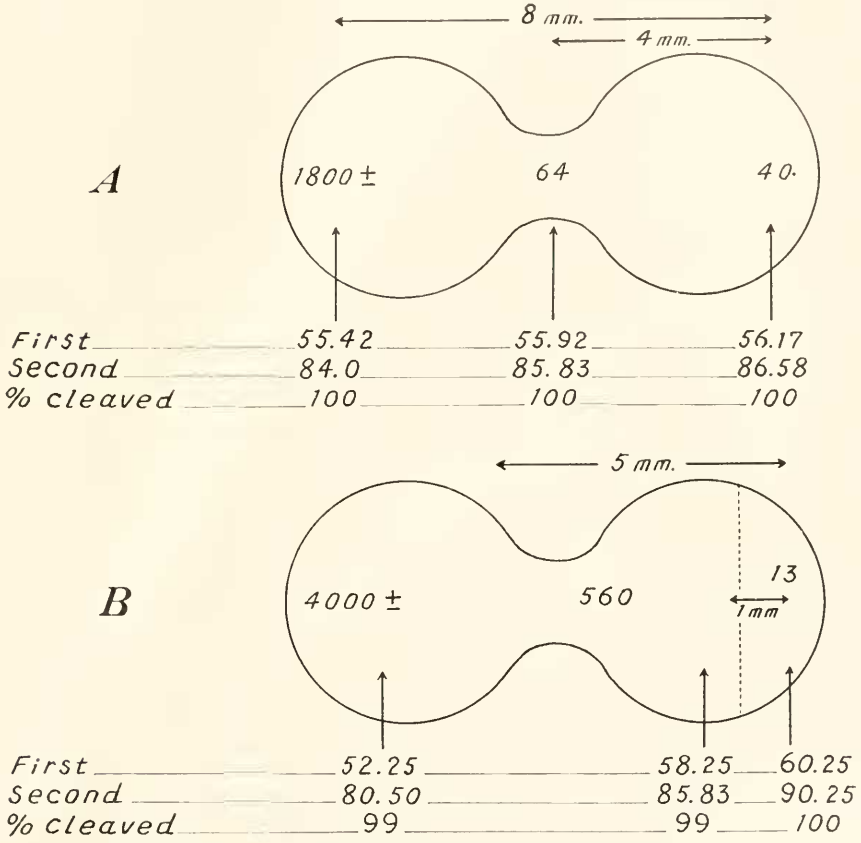

Fig. 1. Diagrams of the connected drops used in two different experiments. Each drop contained 20 cu. mm. The measurements give distances between points indicated by the double arrows. Figures below the diagrams, unless otherwise indicated, give time in minutes to the designated cleavage.

The values just given include those with hanging drops where the relations are obviously different from those obtaining in the other types of experimentation. If the hanging-drop experiments are omitted, these true means become 1.17 and 2.68 minutes with "p" values of 0.016 and 0.0000 respectively. Since these hanging-drop experiments do not support the evidence from the rest of the work to date and since

we do not know why this is the case, it is probably better to include these in the summaries. At least this is the conservative procedure and is in line with the general practise in these experiments of weighting the results against the experimental trend whenever it was impossible to be absolutely impartial.

Thus far in this account we have been dealing mainly with time relations with regard to 50 per cent cleavage. It is profitable to examine the assembled data from another approach which may be indicated by the following question: How many cases were there in which there was evidence of a shorter time elapsing between fertilization and 50 per cent cleavage in the denser population of eggs and in how many cases

TABLE III

Relations between first and second cleavages.

Evidence of Stimulation in Denser Population (D)	Without Hanging Drops	With Hanging Drops
1. D cleaved first in both cleavages	46	48
2. First cleavage a tie; D faster in second	24	27
3. D slower in I; faster in II	5	5
4. Sparse faster in I; second even	5	5
Totals	80	85
Evidence of Retardation in Denser Population		
5. S cleaved first in both cleavages	5	8
6. First cleavage a tie; S faster in II	0	5
7. D faster in I; S faster in II	0	0
8. S slower in I; second, even	1	2
	6	15
9. No observed difference	13	17
Totals	19	32

did the opposite hold true? The answers are given in Table III properly subdivided among the different possibilities. In the preparation of this table, unless there was a clear difference between the time to 50 per cent cleavage such that the difference could not be the result of error in observation, the case was recorded as showing no difference. Table III shows correctly that our data clearly indicate speedier cleavage in the more densely populated drops.

These experiments taken together demonstrate fairly conclusively that there is a difference in time to 50 per cent of first and second cleavage for *Arbacia* eggs in relatively sparse and in relatively dense population. The data collected are clearly statistically significant on this point

for the second cleavage, less so for the first. It is necessary to inquire into the degree of crowding which will bring about this result. Observations on this point were made from time to time during the progress of the experiments. Those obtained later in 1934, using the paired drop technique with one drop densely crowded with eggs closely associated or even actually directly connected by a narrow isthmus with a similar drop which contained but few eggs, are summarized in Table IV.

TABLE IV

Numbers present with relation to the crowding effect.

No. Experiments	Dense No. eggs	Sparse No. eggs	Difference in Fifty Per Cent Cleavage		Probability		Size Drops Cu. Mm.	Eggs Followed Sparse
			I	II	I	II		
12–13	1600+	800−	2.04	2.95	0.0036	0.0014	10–40	10–30
15	65–164	5–24	0.23	1.26	0.7	0.045	20	5–24
14	22–56	5–18	0.08	−0.3	0.38	0.426	20	5–18

In this table the difference in time to 50 per cent first and second cleavages are compared for crowded drops containing over 1,600 eggs as compared with that in more sparsely populated drops containing 800 eggs or less. In reality the listing of these hundreds of eggs in the sparser populations is hardly fair for in all cases the eggs watched in such drops were out of contact with each other while those in the densely populated drops were closely crowded. Under these conditions (which have already been discussed in some detail) the eggs in the more crowded drops cleaved significantly earlier than did those in the accompanying sparsely populated drops of equal size. When the more crowded drop contained but 65–164 in a 20 cu. mm. drop and the accompanying drop held 5–24, the difference in time to first cleavage was insignificant but the denser eggs cleaved slightly sooner at second cleavage and the difference of 1.26 minutes is just within the upper limit for statistical significance. When the population in the more dense drop is still further reduced and that in the sparsely populated drop remains about the same, the difference disappears for both cleavages.

Factors Known to Retard Cleavage

The factors known to retard cleavage which may have operated in one or more of these experiments are:

1. Hypertonic sea water.
2. Lowered temperature.

3. Contamination with cœlomic fluid.
4. Contamination with fragmented eggs.
5. Increased amount of metabolic wastes in the water.

Of these, there was no opportunity for differential contamination with cœlomic fluid in the different parts of a given test. The precautions taken against hypertonicity have already been outlined and while they were not always successful when differential treatment was unavoidable as in the time of setting up of the various drops, especial care was taken to load the experiment against the trend of experimental findings.

As regards temperature differences, when one observer examined the one or two slides holding dense populations and the other handled the half dozen or more with sparser ones, there was the possibility of the former having a slightly higher temperature from the more steady contact with the fingers of the observer. This possibility was eliminated in the tests with the assembled moist chambers which constitute the majority of the experiments reported here. Furthermore, these latter experiments yielded greater and more consistent differences than were found under conditions that might have been suspect, hence there is no evidence that the observed differences are the result of an externally induced temperature differential.

There is, however, a possibility that the high rate of oxidation of the larger number of newly fertilized eggs confined in a small space may produce a temperature differential sufficient to account for the observed results. No tests of this possibility have been made to date. The best evidence that can be cited in its favor is that a slight differential increase in temperature would produce the difference which we have found.

The isolated eggs were definitely favored as regards the presence of fragmented or immature eggs. Allee found it psychologically difficult to select other than good eggs for the relative isolation of the sparse lots while in scooping up from 50 to 8,000 eggs for a more densely populated drop, no such selection was possible. Hence the sparsely populated drops contained less debris both absolutely and proportionally than did those with the large number of eggs.

Overcrowding.—In the most densely populated drops used in the regular experiments, eggs were present in about the proportion of 0.5 cc. of centrifuged eggs (lowest speed with power centrifuge for one minute) to six cc. of water. In Syracuse watch-glasses with this concentration there was reduced cleavage. In drops of 10 or 20 cu. mm., the eggs are nearer to the surface of the drops and while they may be piled three or four deep in the center, cleavage goes almost as well as in the

finger-bowl controls. When the same concentration is placed in a watch-glass or finger-bowl, i.e. in the same concentration when the eggs are all stirred up, the eggs settle to the bottom in a much more dense mass than any we worked with on the slides and under these conditions they were definitely over-crowded.

Mass Protection.—In an attempt to avoid the added evaporation incident to changing clouded covers, a microscope stage-cooling device was developed. This consisted of a brass quadrangle about the length of the microscope stage and somewhat narrower and about three centimeters high. Glass tops and bottoms were sealed on with DeKotinsky cement. The upper glass plate had patches of etched lines to facilitate counting and accommodated two of the 3 cm. glass rings which were sealed to the glass with vaseline. Intake and drain tubes were soldered to the brass quadrangle and were attached to the sea water supply since this was the coolest water available. This was further cooled by running it through a copper coil placed in a bucket of ice water. The drops were placed on the glass as usual except that during isolation, a covering watch-glass was used for a partial moist chamber. After covering the drops with the usual vaseline-sealed, glass cover, the water was turned through the chamber and the temperature fell. With the room temperature at 24°, in one instance, water emerging from the cooling device showed 12°; it was usually held at from 17–19°. This device was discarded when it appeared that there was difficulty in proper cleaning of the surface to which the eggs were exposed and no data secured by this means have been included in the preceding tables.

Some twelve experiments were tried using this gadget. The mean difference to 50 per cent cleavage for those that cleaved was 5.0 minutes for first and 5.62 minutes for second cleavage, with "p" values of 0.024 and 0.0052 respectively. Four accompanying experiments made in the assembled moist chambers already described had differences to 50 per cent first and second cleavages of 0.31 and 2.8 minutes respectively with the latter difference statistically significant. In two cases with the stage-cooling device, sparse populations of 6 and of 10 eggs failed to cleave and in two other experiments sparse lots of 24 and of 34 did not reach 15 per cent cleavage. The accompanying densely populated drops developed to or beyond the blastula stage and in three of the four comparisons just made, many in the more crowded lots were actively swimming after 24 hours. These data strongly suggest the presence of some toxic contaminating substance which was not completely removed by the methods used in washing these stage-cooling devices. Further tests showed that in connected drops gradients of resistance could be demonstrated which depended on the numbers present, the more eggs within

the limits tested, the greater the percentage of cleavage and the further development would proceed before death. Such mass relations appear to be closely related to the mass protection from toxic materials which has been repeatedly demonstrated (*cf.* Allee, 1931, 1934).

Could this have been the explanation of the more rapid cleavage observed in the denser populations? There is a suggestion that it may have been a factor in the paraffined slides, which, however, is not borne out by comparative studies unless there was some other toxic agent acting similarly in the remainder of the experiments. To suppose that there was mass protection from toxic materials in the other experiments would imply either some sort of toxic emanations from the glass itself or from some chemical previously in contact with it (Richards, 1936), or that traces of the vaseline and/or vacuum grease remained over from the washing and were poisonous, or that some of the soap from the mild suds used remained after the extensive and careful rinsing. There is no evidence for the presence of toxic materials from any of these sources all of which were considered as possible means of experimental error before this set of experiments was begun. Direct tests made by using water which had stood over masses of broken glass showed no difference in development as compared with similar cultures in ordinary sea water. Direct tests for toxic effects from vaseline and from the vacuum grease used made by coating slides with these substances and placing drops with different numbers of eggs on them, yielded no evidence of contamination from this source. Hence we concluded (and later experiments to be reported in another paper justify the conclusion) that the differential results obtained are not produced by mass protection such as was demonstrated to be operating in the experiments made with the stage-cooling device.

There is also internal evidence from the experimental results reported in Table II that something more is happening in these experiments than would be expected from the simplest of mass relations whether mass protection or otherwise. The data given there show that the mean time to 50 per cent first cleavage for all the experiments was 0.88 minutes (omitting hanging-drop experiments, 1.17 minutes) and to second cleavage was 2.23 minutes (omitting hanging drops, 2.68 minutes). The mean time to 50 per cent first cleavage was 57 minutes and to the same stage in second cleavage was 85 minutes.

If this were a case of simple mass relationships one would expect the acceleration of 0.88 minutes during a period of 57 minutes to continue at the same rate during the following 28 minutes to second cleavage. At this rate the total acceleration would approximate 1.31 minutes,

which is not the case. Even when one corrects for the fact that for the first ten minutes after fertilization, on the average, the eggs which are to make up the isolated or sparse populations are in fairly dense lots, the expected acceleration on the basis of uniform, simple mass action would be 1.40 which even yet is far from the observed value. Even omitting the hanging-drop experiments, which we decided above not to do, and using the basis of calculation which will give highest results, simple mass action would call for an acceleration by second cleavage of 1.87 against the observed value of 2.68 minutes. These relationships indicate that the observed phenomena are not based directly on the simplest sort of mass physiology.

Other possible causal factors which deserve investigation include the effect, if any, of increased carbon dioxide, of supernatant water from eggs both before and after cleavage, and of mitogenetic rays.

Summary

1. Other conditions being equal and under a variety of experimental conditions, eggs of *Arbacia punctulata* cleave more rapidly when in relatively dense as opposed to relatively sparse populations. The decreased time to first cleavage in the dense populations was 0.88 minutes and to second cleavage was 2.23 minutes. The first difference is probably not statistically significant; the second value is clearly significant.

2. Among other conditions, these relations were observed when some thousands of eggs in a drop of 20 cu. mm. of sea water were connected by a narrow strait with a similar drop holding some few tens or even a few hundreds of eggs.

3. If the eggs were crowded together too densely, the time to first and second divisions was definitely retarded and the percentage of final cleavage was reduced.

4. When 22 to 56 eggs were placed in one drop of 20 cu. mm. connected by a strait with another containing 5 to 18 eggs, no difference in cleavage rate was observed.

5. The observed differences are not a result of differential temperatures externally imposed, differential hypertonicity or hypotonicity, contaminations with cœlomic fluid or with fragmented eggs.

6. Such results may be obtained by mass protection from toxic materials. There is, however, no indication that differences here reported were so caused.

7. Although no supporting evidence is presented here, the results may conceivably have been the result of differential temperatures produced by the high rate of oxidation of the massed eggs in a small space,

by chemical emanations from the eggs, including carbon dioxide, by stimulation from mutual contact or by mitogenetic rays. Discussion of these problems is reserved for the present.

8. In addition to their intrinsic interest, the results provide another instance of physiological activities which proceed more rapidly at an intermediate optimum than when either too few or too many are present.

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